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Inhibition of HIV Protease Activity by Heterodimer Formation†

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ABSTRACT: The dimeric nature of the HIV protease has been exploited to devise a novel mode of inhibiting the enzyme. The use of defective monomers or nonidentical subunits to exchange with wild-type homodimers produces catalytically defective heterodimers. Incubation of the HIV1 or HIV2 protease with a 4-fold molar excess of an inactive mutant of HIV1 leads to 80 and 95% inhibition of enzyme activity, respectively. Incubating HIV1 and HIV2 proteases at a 1:5 ratio results in a 50% reduction of activity of the mixed enzymes. The HIV1/HIV2 heterodimer was identified by ion-exchange HPLC. The heterodimer may display a disordered dimer interface, thereby affecting the catalytic potential of the enzyme. This mechanism of inactivation is an example of a dominant negative mutation that can obliterate the activity of a naturally occurring multisubunit enzyme. Furthermore, it provides an alternative to active-site-directed inhibitors for the development of antiviral agents that target the dimeric interface of the HIV protease.

The homodimeric nature of the protease from the human immunodeficiency virus 1 (HIV1)[1] has been confirmed by X-ray crystallographic (Navia et al., 1989; Wlodawer et al., 1989) and biochemical analysis (Meek et al., 1989). The two N-termini (residues 1-4 and 1′-4′) and two C-termini (residues 94-99 and 94′-99′) of the HIV1 protease dimer form β strands that interdigitate to create a four-stranded antiparallel β sheet (Wlodawer et al., 1989). This β sheet is partially stabilized by intersubunit backbone H bonds of alternate amino acids from each of the four strands (Figure 1A). Approximately 50% of the amino acid residues at the dimer interface with intermolecular distances between 2.5 and 3.3 Å are provided by this arrangement of the four termini.

Assembly of two HIV1 protease monomers results in a dimer of approximately 22000 daltons and generates an active site at the interface of the subunits. Each monomer contributes half of the active site which includes two catalytic aspartic acids as well as threonine/serine and glycine residues, which are conserved among all aspartyl proteases for their structural role in active-site geometry (Pearl & Taylor, 1987). The extensive interface that exists between the monomers is dominated by interactions between adjacent amino and carboxyl termini as well as those between twin amino acids at the active site (Wlodawer et al., 1989). Dimer formation generates not only the catalytic center but also the extended substrate binding pocket.

Viral proteolytic activity is essential for the generation of infectious virus particles in HIV and related retroviruses (Kohl et al., 1988). Autoprocessing of the protease from the *gag* and *gag/pol* polyprotein precursors results in the release of the protease and the generation of mature structural and enzymatic proteins (Debouck et al., 1987; Giam & Boros, 1988; Lillehoj et al., 1988). The pivotal role of the protease in posttranslational processing of viral proteins makes it a prime target for drug design. Agents that specifically and effectively inhibit the hydrolytic activity of the protease may serve as powerful antiviral pharmaceuticals (DesJarlais et al., 1990; McQuade et al., 1990; Meek et al., 1990). The requirement for the protease to dimerize provides an alternative mechanism for inhibiting enzyme activity than active site-directed inactivation. Prevention of the HIV protease homodimer assembly or disruption of the assembled dimer interface should effectively block viral protease catalysis. Inhibition of the heterodimeric herpes virus ribonucleotide reductase by synthetic peptides corresponding to the C-terminus of the small subunit suggests that interference in normal subunit interactions can lead to enzyme inactivation (Cohen et al., 1986; Dutia et al., 1986).

We have previously identified an autolysis product of the HIV1 protease which lacks the N-terminal five amino acids and results from the hydrolysis of a surface-accessible Leu5-

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[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HIV, human immunodeficiency virus; HPLC, high-pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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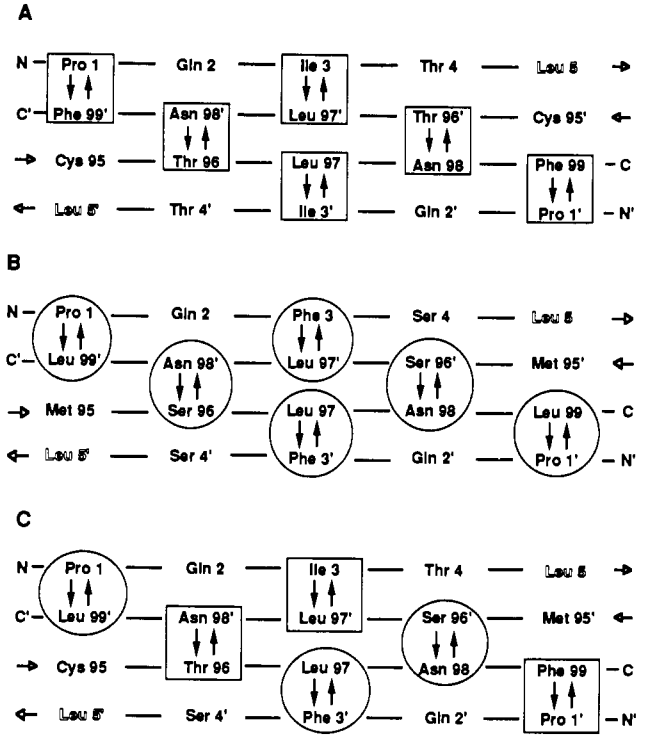

FIGURE 1: N- and C-terminal amino acids involved in the HIV protease dimer interfaces. (Panel A) Alignment according to the three-dimensional structure of HIV1 (Wlodawer et al., 1989). (Panel B) Modeled interface for the amino acid sequence of the HIV2 protease. (Panel C) Modeled interface for a heterodimer of HIV1 and HIV2 proteases. Numbers (1–4, 95–99, 1′–4′, and 95′–99′) refer to amino acid residues for the two polypeptide chains of the dimers. Double arrows indicate hydrogen bonds across backbone atoms. N and C indicate the amino and carboxyl termini, respectively. Squares surround amino acids known to make backbone contacts in the HIV1 structure. The amino acids in the HIV2 sequence which are not identical with the HIV1 sequence but are expected to make backbone contacts are circled.

Trp6 peptide bond (Babé et al., 1990; Pichuantes et al., 1989). The gradual appearance of the 94 amino acid autolysis product, HIVΔ1–5, is concomitant with a decrease in enzymatic activity of the purified protease and may represent a step in the regulation of proteolytic activity in vivo. Presumably, loss of the five N-terminal amino acid residues and the specific backbone contacts at amino acids Pro1–Phe99′ and Ile3–Leu97′ are responsible for loss of enzymatic activity.

Considering the noncovalent nature of the subunit interactions of the enzyme dimer, we expect the existence of a dynamic equilibrium between monomer and dimer structures. Therefore, mixing defective subunits, e.g., HIV1Δ1–5, or homologous subunits, e.g., HIV2, with HIV1 may result in exchange of subunits to generate heterodimeric structures. The heterodimers may display nonnative interfaces that result in catalytically defective dimers and aid in identifying sites in the interface region that are critical for enzyme activity.

Experimental Procedures

Cells and Plasmid Constructions. HIV1 protease was expressed as a 179 amino acid protease precursor fused to human superoxide dismutase in *Escherichia coli* strain D1210 from the plasmid pSOD/PR179 as described previously (Babé et al., 1990). HIV2 protease was expressed and secreted in *Saccharomyces cerevisiae* strain AB110 harboring plasmid pHIV2PR115 as described previously (Pichuantes et al., 1990). Since the HIV1Δ1–5 variant is inactive, it was expressed and secreted in *S. cerevisiae* from plasmid pPR94 which produces a gene product that does not require autoprocessing. This plasmid was constructed by using a 273 bp *Spe*I–*Sal*I DNA fragment from pPR99 (Pichuantes et al., 1989) encoding the 90 C-terminal amino acids of the HIV1 protease and a stop codon. This DNA fragment was ligated to an *Xba*I–*Spe*I synthetic oligonucleotide fragment encoding the *KEX2* processing site (Leu-Asp-Lys-Arg) and amino acids 5–8 (Trp-Gln-Arg-Pro) of the HIV1 protease. The resulting 297 bp *Xba*I–*Sal*I fragment was ligated to the α-factor leader sequences and to the glucose-regulated hybrid promoter ADH2/GAPDH (Barr et al., 1988). The resulting DNA fragments were cloned into the yeast expression vector pBS24.1 (Pichuantes et al., 1989). This vector uses the α-factor terminator (Brake et al., 1984) to ensure transcriptional termination and the yeast genes leu 2-d and ura 3 for selection. The defective leu 2-d allele (Erhart & Hollenberg, 1983) is used to increase the copy number of the plasmid. The 2u sequence which includes two inverted repeats is present for autonomous replication of the plasmid in yeast. The β-lactamase gene and ColE1 origin of replication are present for selection and autonomous replication in *E. coli*. The plasmids were used to transform spheroplasts of the protease-deficient *S. cerevisiae* strain AB110 (MATa, leu2-3 112, ura 3-52, pep4-3, his 4-580 {cir°}). Leucine prototrophs were selected and grown in uracil-deficient media essentially as described previously (Barr et al., 1988).

Protein Purification. The HIV1 protease was purified from bacteria as described previously (Babé et al., 1990). The HIV1Δ1–5 protease was purified from the media of yeast cultures expressing plasmid pPR94 grown 72–96 h. All steps were carried out at 4 °C unless stated otherwise. Following centrifugation of the cells, the culture supernatant (generally 6–12 L) was loaded on a phenyl-Sepharose column (5 × 60 cm, Pharmacia) preequilibrated in 20 mM sodium phosphate, pH 8.0, 1 M ammonium sulfate, 1 mM EDTA, and 1 mM PMSF. The column was washed with a linear gradient of 1–0 M ammonium sulfate in buffer, and the protease was eluted with water. The fractions were concentrated in a dialysis bag over a bed of poly(ethylene glycol). Further purification was achieved by preparative isoelectric focusing on a Rotofor unit (Bio-Rad) using a mixture of 1 mL of pH 3–10 Ampholytes and 0.25 mL of pH 8–10 Ampholytes in a total volume of 55 mL. If the purified protein was not to be used for mass spectrometric or X-ray crystallization trials, Triton X-100 was added to 0.05% final concentration, which increased the yield of purified material by approximately 50%. The protease-containing fractions in the range of pH 8.5–11 were localized by immunoblotting, pooled, and concentrated. These samples were then run on reverse-phase HPLC using a preparative C_3 column (4.6 × 21.2 cm, Dupont) eluted with a linear gradient of 25–85% acetonitrile/water in 0.1% TFA over a period of 60 min at a flow rate of 10 mL/min. The protein eluted at about 60% acetonitrile and is approximately 95% pure as judged by silver stains of samples run on SDS–PAGE. In all cases, the protease was monitored by immunoblots using polyclonal antibodies raised against a *pol* polypeptide (Pichuantes et al., 1989).

The HIV2 protease was purified essentially as described for HIV1Δ1–5, with the following exceptions. The yeast supernatant was adjusted to 1 M ammonium sulfate, 20 mM sodium phosphate, pH 8.0, 1 mM EDTA, and 1 mM PMSF prior to being loaded on a phenyl-Sepharose column equilibrated with the same buffer. The addition of salt was required for binding of the HIV2 protease to the column due to the less hydrophobic nature of the HIV2 enzyme as compared to the HIV1 enzyme (consistent with the lower content of hydrophobic residues in

the HIV2 protease). In the purification step using the Rotofor unit, the pH 8–10 Ampholytes were replaced with pH 5–8 Ampholytes, and the protease migrated to the pH range 5.0–6.5. The HIV2 protease was detected by immunoblots using rabbit polyclonal antibodies raised against a chemically synthesized, 99 amino acid HIV2 polypeptide kindly provided by Drs. I. Kuntz and E. Bradley of UCSF. The anti-HIV2 antibodies do not cross-react with the HIV1 protease using our immunoblotting conditions.

Preincubation of HIV1 and HIV2 Proteases and in Vitro Proteolytic Activity Assays. Both HIV1 and HIV2 proteases were assayed by measuring the rate of specific hydrolysis of the decapeptide Ala-Thr-Leu-Asn-*Phe-Pro*-Ile-Ser-Pro-Trp. The decapeptide corresponds to the sequence of the natural HIV1 gag–pol polyprotein sequence from amino acid 151 to 160 and represents the junction between the protease and the reverse transcriptase (Lightfoote et al., 1986). Cleavage of the synthetic peptide as well as the gag polypeptide occurs at the Phe–Pro peptide bond. The purified HIV1 or HIV2 proteases, 100 ng (250 nM), were incubated for 16 h at 25 °C with 0, 20 (50 nM), 40 (100 nM), 100 (250 nM), 200 (500 nM), and 400 (1 μM) ng of purified HIV1Δ1–5 in 40-μL volumes of assay buffer containing 150 mM sodium acetate, pH 5.5, 10 mM DTT, 2% glycerol, 1% ethylene glycol, and 1 mM EDTA. A 5-μL aliquot of 1 mM decapeptide substrate was then added and the incubation proceeded for 8 h at 25 °C. The reaction was stopped by adding 5 μL of 1% TFA in neat acetonitrile. The samples were fractionated by HPLC using a C_{18} column (0.46 × 25 cm, Vydac) eluted with a linear gradient of 0–60% acetonitrile/water in 0.1% TFA over a period of 20 min at a flow rate of 1.0 mL/min. As described previously (Babé et al., 1990), the conversion of the decapeptide to two pentapeptides by hydrolysis of the Phe–Pro peptide bond was quantitated from the HPLC chromatogram and used to calculate enzyme activity (micrograms of peptide digested per microgram of enzyme per minute). Results are expressed as the percentage of HIV1 or HIV2 activity remaining after incubation with increasing amounts of HIV1Δ1–5. Refolding of HIV1Δ1–5 was accomplished by denaturation in 8 M urea followed by a 10-fold dilution into the assay buffer described above. This procedure has been shown to restore enzymatic activity to preparations of HIV1 (Tomasselli et al., 1990b) and HIV2 (Pichuantes et al., 1990) proteases that have lost activity due to denaturation. Incubations using refolded proteins contained a final urea concentration of 0.2 M, which is below the concentration of urea (0.3 M) shown to affect proteolysis (Tomasselli et al., 1990a).

Mono Q HPLC Separation. A mono Q HR 5/5 column (Pharmacia) was equilibrated with 20 mM sodium phosphate, pH 7.5, and eluted with a linear gradient of 0–1.0 M NaCl in the same buffer at a flow rate of 0.5 mL/min at 25 °C. The elution profile was monitored at 280 nm, and 0.5-mL fractions were collected and assayed by immunoblotting using antibodies against either the HIV1 or the HIV2 protease.

Results and Discussion

Mixing of HIV1 or HIV2 Protease with HIV1Δ1–5. A 250 nM solution of purified HIV1 protease was preincubated for 12–18 h at 25 °C with 0–4 molar equiv of HIV1Δ1–5. The proteolytic activity of the mixtures was measured as described above. The HIV1Δ1–5 protease displayed no detectable enzymatic activity. Incubation of purified samples of the HIV1 protease with increasing amounts of HIV1Δ1–5 leads to a loss of enzymatic activity as shown in Figure 2. A 1:1 molar ratio of HIV1 to HIV1Δ1–5 leads to a 40% loss of HIV1 activity. A proportionally higher 80% loss of activity was achieved with

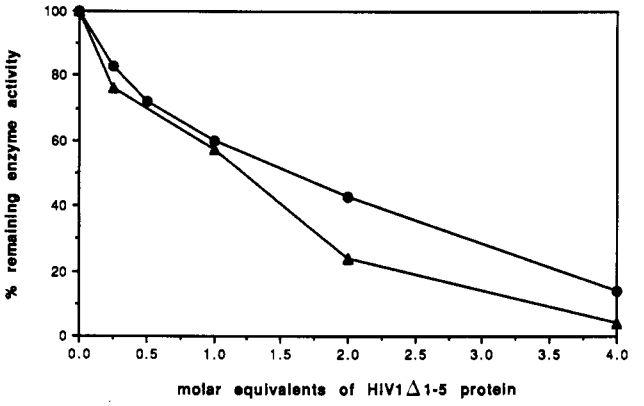

FIGURE 2: Results of mixing HIV1 or HIV2 proteases with increasing amounts of HIV1Δ1–5 polypeptide. HIV1 or HIV2 proteases were incubated with 0–4 molar equiv of HIV1Δ1–5 in 40 μL as described in the text. The peptide substrate (Ala-Thr-Leu-Asn-*Phe-Pro*-Ile-Ser-Pro-Trp) was then added, and the incubation proceeded for 8 h at room temperature. The samples were separated on HPLC, and the conversion of substrate to products was determined in order to calculate enzyme activity (micrograms of peptide digested per microgram of enzyme per minute). Results are expressed as the percentage of HIV1 or HIV2 activity remaining after incubation with increasing amounts of HIV1Δ1–5. (●) HIV1 + HIV1Δ1–5. (▲) HIV2 + HIV1Δ1–5.

a 4 molar excess of HIV1Δ1–5 over HIV1. The extent of inhibition of HIV1 by addition of HIV1Δ1–5 is time-dependent. Preincubation times of 0, 2, 6, and 16 h result in 85, 80, 65, and 20% remaining activity, respectively. This time dependence in conjunction with the proportionally greater extent of HIV1 protease inhibition at higher HIV1Δ1–5 concentrations suggests that the loss of activity is due to the formation of the heterodimer HIV1/HIV1Δ1–5 and that the subunit exchange is relatively slow with respect to our experimental conditions. The precise arrangement of amino acid residues observed in the HIV1 homodimer interface, which maintains the dimer structure and forms the catalytic center, may not form properly in the case of these heterodimers due to the loss of the N-terminal amino acids. The use of an active-site mutant enzyme such as Asp25Asn for heterodimer exchange will help to clarify the significance of the interface contacts and is currently under investigation.

Amino acid sequence alignments for the known HIV and SIV types and subtypes show that within the interface region of the protease dimer the amino acids Pro1, Gln2, Leu5, Trp6, Gly94, Leu97, and Asn98 are strictly conserved. In particular, despite a 55% difference in the amino acid composition of the HIV1 and HIV2 proteases (Guyader et al., 1987; Sanchez-Pescador et al., 1985), similar β-sheet interactions in the HIV2 dimer interface are modeled from the HIV1 structure (Figure 1B, unpublished results). If the overall protein fold is maintained between the HIV1 and HIV2 proteases, a similar interface is expected for both viral proteases, and the two proteases may exchange to yield heterodimers. A 250 nM solution of purified HIV2 protease was preincubated for 12–18 h at 25 °C with 0–4 molar equiv of HIV1Δ1–5 and analyzed as described above. Similar to what was observed for the HIV1 protease, increasing concentrations of the HIV1Δ1–5 result in a concomitant decrease in HIV2 protease activity (Figure 2). The slightly greater inhibition of the HIV2 protease with HIV1Δ1–5 than inhibition of the HIV1 protease may represent a stronger association of the HIV1 homodimer than of the HIV2 homodimer. This potentially stronger association of the HIV1/HIV2 heterodimer is also reflected in the extent of inhibition with respect to the preincubation times: 0, 2, 6, and 16 h of preincubation results in 43, 35, 30, and 4% remaining

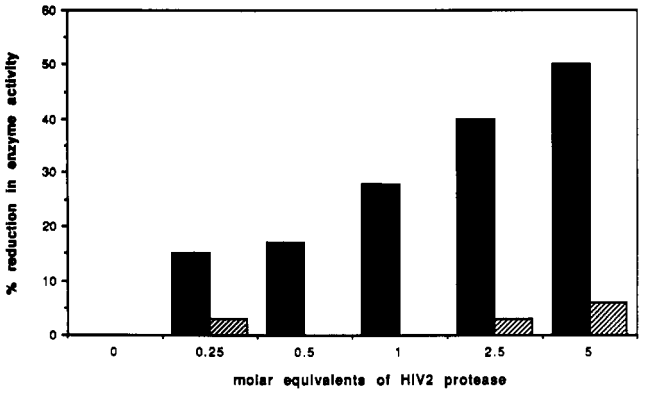

FIGURE 3: Results of mixing HIV1 and HIV2 proteases. The enzymes were incubated, and the enzymatic activity was monitored as described in the text. Since the substrate used is cleaved by both enzymes (albeit more efficiently by HIV1), samples of HIV1 and HIV2 were preincubated separately and assayed for activity in order to calculate turnover rates for each enzyme. The sum of these values constituted the expected wild-type activity (set to 100%). The values obtained for the mixed samples were compared to the expected activity, and the difference is presented as the percent reduction in activity for the different mixtures. Solid bars, HIV1 + native HIV2; hatched bars, HIV1 + denatured (inactive) HIV2.

enzyme activity, respectively. Determination of the various homo- and heterodimer association constants is currently underway.

Mixing of HIV1 and HIV2 Proteases. The effects on enzyme activity by heterodimer formation between HIV1 and HIV2 proteases were monitored by hydrolysis of the decapeptide substrate Ala-Thr-Leu-Asn-*Phe-Pro*-Ile-Ser-Pro-Trp. This substrate represents the protease–reverse transcriptase junction sequence of the HIV1 gag–pol precursor and is approximately 5 times more specific for HIV1 than for the HIV2 protease under our assay conditions. A steady decline in the expected activity of the HIV1 and HIV2 enzymes was observed as increasing amounts of HIV2 were added, achieving a level of 50% loss of activity with a 5-fold molar excess of HIV2 over HIV1 (Figure 3). The expected wild-type activity (100%) was calculated as the sum of the values obtained for the two enzymes when incubated separately. Since the concentration of peptide present was 100–500-fold greater than the concentration of HIV1 and HIV2 enzymes, the loss of activity cannot be due merely to sequestration of peptide. Furthermore, the loss of activity is not due to autodegradation since immunoblot analysis of reaction mixtures did not show significant degradation of either HIV1 or HIV2 protease during the course of the reactions.

A heat-denatured and inactive preparation of HIV2 protease does not significantly inhibit the HIV1 protease activity when incubated with HIV1 homodimer. A maximum of 5% reduction in HIV1 protease activity was observed with 5 molar equiv of denatured HIV2 protease (Figure 3). This indicates that only folded monomers are capable of exchange and formation of heterodimers and illustrates the requirement of a properly folded monomer for the association of dimers. The inactive HIV2 preparation could then be denatured in urea, refolded to yield an active enzyme, and shown to inhibit HIV1 activity upon mixing (data not shown).

Comparison of the amino acid sequences of the two enzymes reveals that the majority of the amino acids at or near the active site are identical, while several mostly conservative changes are located in the regions involved in dimer contacts (Figure 1C). The amino acid differences presumably perturb amino acid side chain packing at the HIV1/HIV2 heterodimer interface. The substitution of Ile to Phe at position 3 and that of Cys to Met at position 95 may be significant, considering that the side chains of both Ile and Cys are buried in the HIV1 structure (Lapatto et al., 1989). Recent modeling of an HIV1/2 chimeric dimer has also implicated the Phe99Leu substitution in the generation of asymmetric hydrophobic interactions in the heterodimer; together, these changes may reduce the stability of the heterodimer when compared to either homodimer (A. Gustchina and I. Weber, personal communication).

Unlike other proteins, such as the *trp* aporepressor, where heterodimer formation in vitro required thermal induction (Graddis et al., 1988), the HIV protease monomers may exchange in the absence of significant unfolding. A series of nonionic detergents show strong inactivating effects on the HIV2 protease when tested in the peptide assay at their critical micellar concentrations. Incubations with octyl β-D-glucopyranoside (20 mM), octyl β-D-thioglucopyranoside (10 mM), or octanoyl *N*-methylglucamide (60 mM) lead to an 85–95% loss of enzyme activity. This loss of activity can be easily reversed by dialysis to remove the detergent, suggesting that the enzyme dimers undergo facile dissociation and reassociation depending on the environment. The zwitterionic detergent CHAPS (7 mM) has a limited effect (25% reduction of activity), while polyoxyethylenes such as Triton X-100 (0.5 mM) and Tween-20 (0.05 mM) have no inhibitory effect on the HIV2 protease activity possibly due to their larger size.

Disruption of the homodimeric interface region of a related viral protease with polypeptides has been shown to lead to inactivation of the enzyme, illustrating the sensitivity of this region and the potential for inhibition by disruption of dimer structures. The enzymatic activity of the avian myeloblastosis virus protease was reduced in vitro by addition of protease fragments generated by cyanogen bromide digestion, implying that homodimer formation might be competitively inhibited by the fragments (Katoh et al., 1989). The requirement for a 30-fold excess of CNBr fragments to intact protein relative to our equimolar ratios of polypeptides emphasizes the importance of a nativelike structure to disrupt the heterodimer. These results do not preclude the use of synthetic molecules that may structurally mimic the interface region and serve as inhibitors of dimerization, as well as the use of active-site-defective mutant proteases to create inactive heterodimers.

Identification of Protease Heterodimers. The extensive amino acid differences between the HIV1 and HIV2 proteases results in a dramatic difference in charge. The isoelectric points calculated for the HIV1 and HIV2 proteases are 9.9 and 5.1, respectively, and are in agreement with the values we have determined from the preparative isoelectric focusing purification step discussed earlier. The formation of a heterodimer between these two species can be monitored by identifying new species with isoelectric points of intermediate values. Such species have been identified by HPLC separation on an ion-exchange column (Figure 4). At pH 7.5, the HIV1 protease is not retained by an anion-exchange column (Figure 4A1), while the HIV2 protease binds tightly and elutes with 1 M NaCl (Figure 4A2). Preincubation of the proteases for 16 h in equimolar ratios results in the formation of new protein species which elute between 250 and 500 mM NaCl (Figure 4A3). Immunological analysis of the fractions from the HIV1/HIV2 protease mixture reveals that these new species react with antibodies specific for both HIV1 and HIV2 proteases (Figure 4B). The heterogeneity of the intermediate peaks may represent slight charge and/or size differences caused by proteolysis of the homodimeric and heterodimeric species. Isoelectric focusing reveals charge heterogeneity for

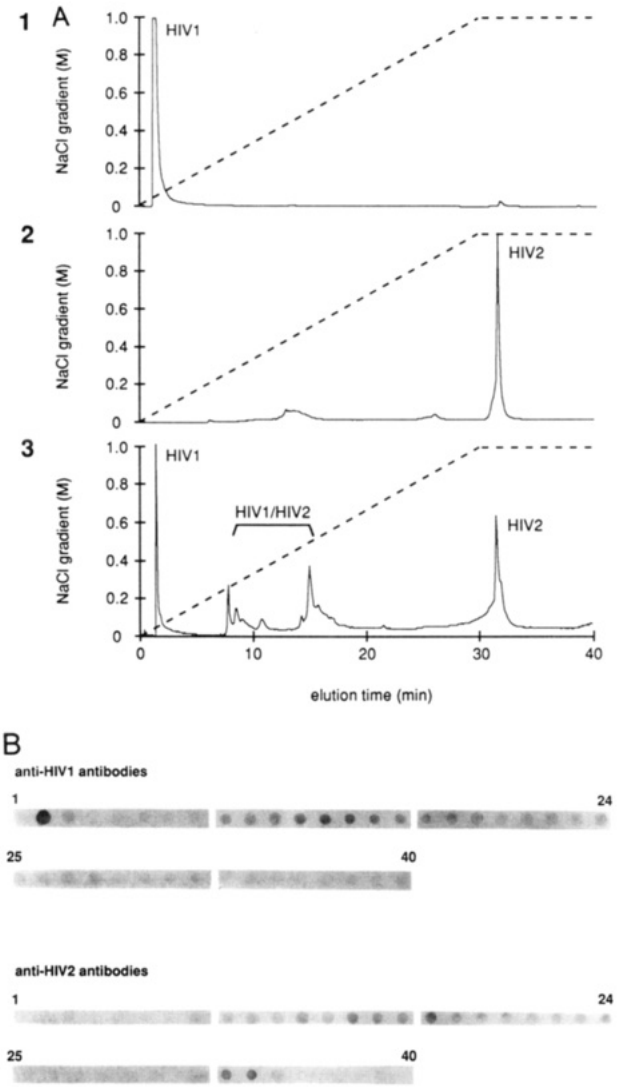

FIGURE 4: Identification of HIV1/HIV2 protease dimers resulting from mixing equal amounts of purified enzymes. (Panel A) A Mono Q HR 5/5 FPLC column was equilibrated with 20 mM sodium phosphate buffer, pH 7.5, and eluted with a linear gradient of 0–1 M NaCl in the same buffer at a flow rate of 0.5 mL/min. The elution profile was monitored at 280 nm, and 0.5-mL fractions were collected. 1, HIV1 protease, elution time 1.0–2.5 min; 2, HIV2 protease, elution time 29.0–33.0 min; 3, HIV1 and HIV2 proteases in equimolar amounts (5 μg), preincubated for 16 h at 25 °C in 100 μL of assay buffer (150 mM sodium acetate, pH 5.5, 10 mM DTT, 2% glycerol, 1% ethylene glycol, and 1 mM EDTA); elution time for the new species 7.0–17.0 min. The above elution times define the regions integrated for calculation of peak areas. (Panel B) Dot-blot immunological detection of either HIV1 (1) or HIV2 (2) proteases. Fractions from the HPLC separation shown in Panel A3 where applied to nitrocellulose and probed with antibodies specific for either protease.

the intermediates formed upon heterodimer formation (data not shown). These species constitute approximately 30% of the HIV1/HV2 protein mixture as judged by the integration of the protein peaks. This is in agreement with the near 30% inhibition of activity observed for the equimolar mixture of HIV1 and HIV2 proteases (Figure 3).

Functional inactivation of a gene by dominant negative mutations can occur by the overexpression of a mutant polypeptide that when overexpressed disrupts the activity of the wild-type gene (Herskowitz, 1987). In the case of oligomeric enzymes, the introduction of a mutant monomer that interacts with a wild-type monomer can result in an inactive multimer. Recently, this approach was applied to the Rev protein of the HIV1 virus (Malim et al., 1989). Just as the trans-activating activity of Rev could be inhibited by the introduction of a defective mutant in vivo, so is the protease inhibited by introducing mutant polypeptides capable of forming inactive heterodimers in vitro. Thus far, our results show that inhibition and subunit exchange between different protease monomers occur in vitro. Presumably, protease inhibition due to subunit exchange between different protease monomers could also occur in vivo.

There are several reasons for focusing on the dimer interface as a site for antiproteolytic intervention. First, dimerization of the protease polypeptides is essential for activity and is also one of the initial posttranslational steps. Second, the extended interface created by the N- and C-termini may be less vulnerable to mutational escape than the active site or the substrate binding regions of the protease and therefore maintained in a greater number of evolving viral strains. Third, comparison of the 3D structure and amino acid composition of the Rous sarcoma virus (RSV) protease and the HIV1 protease reveals that most amino acid residues involved in the intermolecular interface are conserved and the fold is nearly superimposable (Miller et al., 1989; Weber et al., 1989). Therefore, targeting the interface may result in the design of broader acting inhibitors capable of blocking dimerization of a wide range of retroviral proteases. The effects of specific mutations on the formation of active dimers may identify those amino acids most important for dimer formation and stability and may suggest other venues for dimer inhibition in vivo.

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Electronic Energy Transfer and Fluorescence Quenching in the Active Sites of Mercuric Reductase†

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ABSTRACT: The FAD-containing enzyme mercuric reductase has been studied by means of steady-state and time-resolved fluorescence spectroscopy. The fluorescence relaxation of the excited state of the isoalloxazine ring of FAD can be described by a sum of two exponential functions. The two lifetimes are not due to a different lifetime of each of the two FAD molecules of mercuric reductase. The FAD molecules are quenched dynamically by a quencher that is not sensitive to the solvent viscosity. In vitro activation induces a dynamic quenching of fluorescence, while upon binding of $NADP^+$ the FAD molecules are both statically and dynamically quenched. Time-resolved fluorescence anisotropy experiments of mercuric reductase in water show that the isoalloxazine ring probably undergoes a rapid and restricted vibrational motion of small amplitude. Electronic energy transfer occurs between the two FAD molecules at a rate of about 3.4×10^7 s^{-1}. The angle between the emission transition dipole of the donor and the absorption transition dipole of the acceptor is $137 \pm 2°$ (or $43 \pm 2°$). From previous X-ray data of glutathione reductase we find that the corresponding angle is 160°. This suggests that the isoalloxazine rings of mercuric reductase and glutathione reductase are mutually tilted in slightly different ways.

Resistance to inorganic mercury in many bacteria is mediated via the flavoenzyme mercuric reductase (MR)[1] that catalyzes a two-electron reduction of mercuric ions to elemental mercury using NADPH as the electron donor. MR is a dimer with two identical subunits. Each subunit contains an FAD molecule in close contact with a redox-active disulfide group. The active enzyme has many active-site features in common with a family of disulfide oxidoreductases including

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[1] Abbreviations: MR, mercuric reductase; GR, glutathione reductase; FWHM, full width at half-maximum.

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